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(54) Title: METHOD OF REDUCING COMPLEX CARBOHYDRATES IN FERMENTATION PRODUCTS

(57) Abstract

There are described novel macerating enzyme complexes and methods of using such complexes during a fermentation resulting in improved fermentation and fermentation product. The macerating enzyme complexes of the present invention comprise hemicellulase, cellulase and/or pectinase activity.

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METHOD OF REDUCING COMPLEX CARBOHYDRATES IN FERMENTATION PRODUCTS

Field of the Invention:

This invention relates generally to the treatment of fermentation medium and more particularly to the enzymatic treatment of soy-based fermentation medium to reduce the viscosity throughout the fermentation process and to generally provide benefits in the recovery of the product of such fermentation.

Background of the Invention:

The choice of nutrients used in microbial fermentations can be both a tedious and confusing issue. A nutrient which gives clean fermentations may not be the choice for maximum productivity. On the other hand, those nutrients which give the best productivity, may lead to other problems either in the fermentation or in the recovery of the desired product. For example, one such problem encountered by the fermentation industry is the processing difficulty associated with the use of soy beans as the primary nitrogen source. The problem encountered with the use of soy bean feedstock stems from the high level of carbohydrates associated with the soy bean. An approximate carbohydrate composition of defatted soybean meal is as follows:

Polysaccharides	15-17%
Acidic polysaccharides	8%
Arabinogalactans	5%
Arabinan	1%
Cellulose	1%
Oligosaccharides	14%
Sucrose	7%
Stachyose	5%
Raffinose	2%
Verbascose	trace

Acidic polysaccharides, also referred to as pectins, which comprise about 8% of the polysaccharide portion of the carbohydrate composition of soymeal, are composed of alternating regions of homopolygalacturonans and rhamnogalacturonans. Arabinogalactans and xyloglucans comprise the hemicellulose fraction which are associated with the structural and storage components of soy bean.

The problems encountered from carbohydrate fractions such as described above come in three general areas: 1) the inability to achieve acceptable oxygen transfer capabilities in fermentation due to the increase in viscosity of the medium after sterilization of a soy-based medium; 2) the decrease in flux through ultrafiltration membranes apparently caused by the pectin fractions (rhamnogalacturonans) in the soy; and 3) the precipitation of the pectinic compounds when resulting product is added to heavy-duty liquid detergents which have a low available water content and a highly chelating environment. All these problems need to be addressed to run an enzyme manufacturing process well, particularly at industrial scale.

Accordingly, it is desirable from a commercial manufacturing perspective to develop a method and compositions to address the handling problems which arise from the use of carbohydrate-rich fermentation medium, such as soy meal, in a cost effective manner.

Therefore, it is an object of the present invention to provide enzymatic methods and macerating enzyme complexes to reduce the viscosity encountered in the fermentation process, to increase overall mass transfer in the fermentation, to help alleviate the negative impact of carbohydrate mass on ultrafiltration membranes and to improve the quality of resulting products. Furthermore, it is an object of this invention to provide enzymatic methods and macerating enzyme complexes to increase the flux rates in ultrafiltration and decrease the frequency of membrane fouling in ultrafiltration systems. While these enzyme complexes are all closely related, it is understood that the specific enzymatic activity (or mixture of enzyme activity) may vary depending on the specific fermentation or recovery problem one is attempting to address.

Summary of the Invention:

One embodiment of the present invention provides a macerating enzyme complex or composition characterized by its ability to decrease the viscosity in carbohydrate-rich

fermentations, particularly soy-based fermentations, and/or its ability to increase the overall mass transfer (KLa) in carbohydrate-rich fermentations, particularly soy-based fermentations. A preferred viscosity reducing, mass transfer enhancing, enzyme complex comprises an effective amount of a hemicellulase and cellulase enzyme or components thereof including endogluconases, cellobiohydrolases, β-glucosidase and zylanases, alone or in combination. Preferably such complex substantially comprises hemicellulase and cellulase activity present at a 1X level (dosage) concentration as defined herein.

In another composition embodiment of the present invention there is provided a macerating enzyme complex or composition characterized by its ability to increase flux rates during ultrafiltration in the enzyme recovery phase and/or its ability to decrease the frequency of membrane fouling during ultrafiltration. A preferred enzyme complex for enhancing overall the recovery process, and particularly an ultrafiltration step in such recovery process, comprises enzymes exhibiting pectinase activity, including but not limited to pectin lyase, pectin methylesterase and polygalacturonase. Preferably such enzyme complex substantially comprises pectinase activity present at 1X level (dosage) concentration as defined herein.

In yet another composition embodiment of the present invention there is provided a macerating enzyme complex or composition characterized by its ability to decrease the precipitation of gelatinous substances (pectic substances) when soy-based fermentation products are incorporated in liquid detergent products. The enzymatic complexes of this aspect of the invention comprise enzymes exhibiting hemicellulase activity, alone or in combination with cellulase or pectinase activity. Preferably such complex substantially comprises hemicellulase activity present at a 1X level (dosage) concentration as defined herein.

In a process aspect of the present invention there is provided a process for improving carbohydrate-rich

fermentations and recovery of a desired enzyme product from said carbohydrate-rich fermentations, the method comprising:

a) contacting a fermentation medium high in carbohydrates with an effective amount of one or more macerating enzyme complex;

b) allowing the enzyme complex to act on the fermentation medium until a desired viscosity is achieved;

c) inoculating the fermentation medium of step b) under suitable operating conditions;

d) fermenting the medium of step c) under appropriate conditions; and

e) recovering the desired product.

In a preferred embodiment of this process, the carbohydrate-rich medium is a soy-based medium such as soymeal. Furthermore, the enzyme complexes useful for reducing viscosity substantially comprise hemicellulase and cellulase activity including cellulase enzyme components, while the enzyme complexes useful for addressing recovery (downstream) problems such as membrane fouling comprise pectinase activity, and precipitation of the product when incorporated into a liquid detergent formulation substantially comprise hemicellulase activity. As used herein, substantially comprising either hemicellulase/cellulase or pectinase means that the enzyme complex contains these particular enzymatic activities at least in the amount measured in a 1X concentration dosage as defined in Example 1. For example, substantially comprising pectinase activity means containing at least about 2970 APPV units, regardless of what other enzymatic activity may be present in the complex.

In a preferred embodiment of the present invention the desired product of the fermentation is a protease enzyme, such as a subtilisin, although this invention is applicable to any desired fermentation product which is made using a carbohydrate-rich fermentation medium.

Brief Description of the Drawings:

Figure 1 shows the effect on dissolved oxygen with and without enzyme complex treatment.

Figure 2 shows the effect on mass transfer (KLa) with and without enzyme complex treatment.

Figure 3 shows the absence of correlation of liquid detergent precipitate to glucose as measured by HPLC. Units for glucose from HPLC and precipitate from detergent are arbitrary.

Figure 4 shows the correlation of liquid detergent precipitate to galactose as measured by HPLC. Units for galactose from HPLC and weight of precipitate from liquid detergent are arbitrary.

Figure 5 shows the dramatic decrease in precipitate (as measured by galactose) upon treatment with an enzyme complex.

Detailed Description of the Invention:

Traditionally, complex carbohydrate-protein sources, in particular soymeal, have been used as efficient fermentation raw materials. Soymeal as a nutrient source in a fermentation process has been commercially favorable. However, one of the complications to the use of complex carbon/nitrogen source is the high levels of carbohydrate polymers often present in the raw material but not broken down and utilized by all varieties of organisms. Therefore, an effective breakdown of such polymer present in many types of fermentation raw materials such as casein, meat, animal tissue, collagen, gelatin, corn, cottonseed, egg albumin, soy, skim milk, biomass, etc., is desirable.

Ideally, the fermentation broth at the end of the fermentation should be a clear solution of the product and a separate layer of the producing organisms. In practice, however, the broth often also contains residues of medium components and side products. Therefore, the main purpose of the downstream processing is to purify and concentrate the product, keeping it in a stable form. However, there is a strong interaction between the upstream, e.g., medium

preparation, fermentation, and downstream processes. A fermentation that leaves large amounts of unutilized components at the end of the process, causes serious problems in the downstream section. In other words, the overall process goal is to minimize the total production cost, keeping product quality high.

It is understood that in addition to the carbohydrate-rich protein source, additional fermentation nutrients will be needed, these include but are not limited to phosphate, sulfate, carbonate, bicarbonate, chloride, nitrate, sodium, potassium, ammonium, urea, magnesium, ferrous, calcium, manganese, copper, cobalt, zinc, etc. These nutrients along with soy bean powder are blended in water. This mixture, known as fermentation medium, is heat-sterilized to kill the bacteria. The time, temperature, pH, and macerating enzyme complex activities, after sterilization, are controlled to provide the desired reaction treatment to the fermentation medium.

After one or more enzyme complex treatment(s), the medium is subjected to fermentation by seeding with the desired culture at controlled time (20 to 120 hours), temperature (30°C to 40°C), pH (6.5 to 8), oxygen (0 to 100%) and other nutrient conditions (such as phosphate, sulfate, nitrate, ammonium, magnesium, ferrous, calcium, manganese, copper, cobalt and zinc, but not necessarily additional soymeal added to the medium). It is understood by those skilled in the art that depending on the desired fermentation product, certain conditions will be preferred. See generally, Biochemical Engineering and Biotechnology Handbook, Eds. Atkinson, B. and Mavituna, F., The Nature Press, New York, 1991.

At the end of fermentation reaction, the cells and the other suspended solids are removed by centrifugation or filtration, and dissolved protein product/enzymes are concentrated by ultrafiltration. Water flux across the ultrafiltration membrane is higher if the fermentation reaction has been carried out in the nutrient medium treated with the macerating enzymes. Specifically, treatment with an

appropriate enzyme complex can result in increased membrane flux rate of about 50 to 80%.

Commercial fermentation processes such as for enzyme biosynthesis are generally mass transfer intensive, i.e., nutrients, including oxygen, must be made available to the metabolically active cell population at a high rate. In order to maintain aerobic conditions and high rate of oxygen transfer, fermentation medium viscosity must be low. We have found that by treating a carbohydrate-rich fermentation medium with the macerating enzyme complexes of the present invention a higher dissolved oxygen concentration and a higher oxygen transfer rate is achieved, which results in higher productivity. Importantly, the maceration reduces the fermentation medium viscosity without radically altering the protein's conformation.

The dissolved oxygen (DO) concentration is measured by a polarographic probe used in a state-of-the-art fermentor. The dissolved oxygen probe measures percentage of oxygen present in the fermentation medium. One hundred percent dissolved oxygen represents concentration of oxygen in the fermentation medium when air is being sparged through the medium in which no fermentation reaction is taking place, at 1 bar pressure. Figure 1 shows the effect on DO by treating the fermentation medium with macerating enzyme complexes of the present invention. The dissolved oxygen profiles in Figure 1 are from Bacillus fermentations in a soy bean-based medium, with and without macerating enzyme treatment.

The oxygen transfer coefficient (KLa) represents the fermentation medium capability to transfer oxygen to respiring microorganisms, and is calculated by measuring the dissolved oxygen (DO) concentration as described in Figure 1, and the oxygen uptake rate (OUR). The oxygen uptake rate is calculated by measuring the change in oxygen concentration in the inflow and outflow gas streams of a fermentor. KLa is equal to OUR in mM/h, divided by $((\%O_2)/100*(BP+1)-(\%DO)*0.00418)$ where %DO is 100 when air, containing 20.9% oxygen, is sparged into the fermentor at no reaction and at 1

bar pressure. (BP is back pressure in bar and $\%O_2$ is measured concentration of oxygen in the outflow gas.) Figure 2 shows the effect on KLa by treating the fermentation medium with macerating enzyme complexes of the present invention. The KLa profiles shown in Figure 2 are from Bacillus fermentations in a soy bean-based medium, with and without macerating enzyme treatment.

As used herein, "maceration" means limited degradation such as breakdown resulting in increased flux, increased clarity, reduced viscosity, etc., as compared to "liquefaction" which typically refers to total solubilization of plant cell wall.

The macerating enzyme complexes of the present invention comprise enzymes having cellulase, hemicellulase and pectinase activity or a mixture thereof, and are generally classified as carbohydrases. Carbohydrases normally contain a multi-enzyme system in which varied activities are present. For example, cellulases produced from Trichoderma comprise a complex of enzymatic components including endogluconases, cellobiohydrolases, β-glucosidases and xylanases. Carbohydrases are produced from fungi such as Aspergillus, Trichoderma and Penicillium strains, particularly A. niger, A. oryzae, T. longibrachiatum, P. emersonii, P. funicullosum and others. It is contemplated that any of such carbohydrases having cellulase, hemicellulase and/or pectinase activity would be useful in the present invention.

The following commercially available carbohydrase products were tested for use in the present invention. The products are listed by the commercial supplier, followed by the primary activity of the tested product, where the primary activity is defined by the practitioners, skilled in the art of enzyme applications for primary substrates such as hemicellulose, cellulose and pectins. Since many of the commercially available cellulase, hemicellulase and pectinase enzymes (or mixtures thereof) are associated with different assays for determining specific activities, etc., we have standardized the dosage recommendations for the following

examples based on viscosity units. This standardization was done using Cytolase 104, commercially available from Genencor International, Inc. This standardization is described in detail in Example 1. To the extent that any enzyme complex has comparable activity (1X concentration) in the various viscosity reduction assays described in Example 1, such enzymes (commercially available or otherwise) are encompassed within the present invention.

Products Tested (all with published data on activities):

Product	Supplier	Primary Activity
Sumizyme AC	Sumitomo	Hemicellulase
Sumizyme AP-2	Sumitomo	Pectinase with high polygalacturonase and esterase activities
Cytolase 123	Genencor Intl.	Cellulase with minor hemicellulase activity
Cytolase 177	Genencor Intl.	Pectinase
Cytolase 104	Genencor Intl.	Cellulase, hemicellulase and pectinase activities
Pectinex Ultra-SPL	Novo-Nordisk	Pectinase with some hemicellulase and endoarabinase activities
Cytolase 917	Genencor Intl.	Hemicellulase with minor cellulase activity
PCL5	Genencor Intl.	Pectinase
Pearex	Miles-Solvay	Pectinase, endoarabinase
Clarex	Miles-Solvay	Pectinase
AC/123	Sumitomo/Genencor	Hemicellulase and cellulase

Preferred enzyme complexes of the present invention comprise pectinase activity, cellulase activity and hemicellulase activity. Pectinase products useful in the present invention include but are not limited to commercially available products selected from the group consisting of PCL5, Pectinex Ultra-SPL, Cytolase 177, Sumizyme AP-2, Pearex and

Clarex. Cellulase products preferred in the present invention include but are not limited to commercially available products selected from the group Cytolase 123 and Cytolase 104, and hemicellulase products include Cytolase 917 and Sumizyme AC. Preferably a mixture of these products is used. It is understood that any cellulase, hemicellulase and/or pectinase enzyme mixture/composition can be used in the present invention and that the inventors do not intend the invention to be limited to any extent based on the following examples which demonstrate the utility of commercially available products.

METHODS

1) Biopolymer Assay: A pre-sterilized (121°C) soymeal solution, pH 4.5, was used as the substrate in shake flask studies. The flasks were set at 50°C and treated with the enzyme(s) for 90 minutes at which time the broth was checked for viscosity and the supernatant was treated with a flocculent and spun. The resultant supernatant was treated with 95% ethanol and the precipitants were analyzed for biopolymer using the phenol/sulfuric acid assay (Dubois, M., Colorometric Method for Determination of Sugars and Related Substances, Anal. Chem., 28:350-6, 1956).

2) Viscosities: Viscosities were measured on a Brookfield viscometer, model DV-1, with spindle #2 at 60 rpm.

3) Reducing Sugars Assay: Reducing sugars were measured using para-hydroxybenzoic acid hydrazide (PAHBAH). The reagent was made by mixing 4 volumes of 0.5 M NaOH with 1 volume of 5% PAHBAH in 0.5 M HCl. The reaction mixture consisted of 500$\mu\ell$ of the carbohydrate solution mixed with 1.5 ml of the PAHBAH reagent. The reaction mixture was heated in a boiling water bath for 10 minutes and the absorbance was read off an HP diode array spectrophotometer at 410 nm (D-glucose as standard).

4) Galactose: Galactose was measured on samples using the kit assay supplied by Boehringer Mannheim, which is based on the reduction of NAD by galactose dehydrogenase in the presence of D-galactose.

5) HPLC-SEC: HPLC (size exclusion) analysis was done on a Spectra-Physics system consisting of a SP8800 ternary pump, an 8880 autosampler and a Chrom-jet integrator. Detection was carried out on a Rainin model RI-1 refractive index detector. The column was a Beckman Ultraspherogel SEC-2000, using de-gassed HPLC water at 37°C. Known molecular weight standards were run to determine retention times.

6) Flux Determinations: Flux data were generated using broth from a sterilized fermentation in which a soy media was treated with enzyme(s) for 90 minutes at 50°C, pH 4.5. A flocculent (5% final concentration) was added to each sample and the broth spun to remove solids and biopolymer. The centrate was filtered through a 0.45μ filter and then passed through an ultrafiltration unit. An Amicon Model 3 ultrafiltration unit (0.89 cm^2 membrane area) polysulfone 10k membrane was used and a 1 ml pipette was attached to the permeate line to measure permeate volume over time. Flux was calculated as permeate volume per unit time per unit membrane area.

7) Substrate for HPLC Studies: Substrate was isolated from pre-sterilized (121°C) soymeal media in which the solids-free supernatant was treated with cold 95% EtOH and kept at 0°C for 30 minutes. The precipitate was spun down and resuspended in 50 mM sodium acetate buffer, pH 4.5, for shake flask studies.

EXAMPLE I

Effect of Certain Enzyme Activity on Substrate

Following the methods described in Biopolymer Assay, above, the following tests were conducted at a predefined 1X dosage, based on certain percentages (by weight) of the individual enzymatically active components of the blend, versus the total reaction mixture volume. Thus, those components with hemicellulase activity, such as Sumizyme AC, when added at the same 1X concentration would be at 0.007% (weight/volume), the major pectinase activity products at 0.0184% (weight/volume) and the cellulase products at 0.11%

(weight/volume). Each enzyme component is expressed in viscosity reduction units, e.g., pectinase activity is expressed in units of Apple Pomace Pectin Viscosity (APPV) where each unit is defined as reduction of the viscosity of a standardized apple pectin solution at pH 3.8 and temperature 22°C, measured in a capillary viscometer. Similarly, hemicellulase activity is expressed in units of Locust Bean Gum Viscosity (LBGV) and measured by locust bean gum substrate's viscosity reduction at pH 4.8 and 22°C. Finally, cellulase activity is expressed in Carboxy Methyl Cellulose Viscosity (CMCV) units, measured by viscosity reduction of carbony methyl cellulose at pH 4.8 and 22°C. Therefore, when added at 1X concentration, pectinase activity is about 2970 APPV units, hemicellulase is about 735 LBGV units and cellulase is about 435 CMCV units per liter fermentation medium. The results of this 1X dosage study are shown in Table I-A. The test was repeated at a 5X dosage and the results are shown in Table I-B.

Table I-A

Effect of Enzyme Activity on Residual Biopolymer (1X Dose)

Activity	Viscosity (cp)	Biopolymer (g/l)
Control (no enzyme)	206	4.45
Sumizyme AC	172	3.61
Cytolase 123	160	4.70
Sumizyme AP-2	201	5.09
Cytolase 177	198	4.13
Cytolase 104	110	2.68

Table I-B

Effect of Enzyme Activity on Residual Biopolymer (5X Dosage)

Activity	Viscosity (cp)	Biopolymer (g/l)
Sumizyme AC	104	2.92
Cytolase 123	170	5.00
Sumizyme AP-2	170	0.90
Cytolase 177	167	1.10

Discussion:

In the 1X dosage study (Table I-A), the only apparent significant drop in viscosity resulted from the hemicellulase and cellulase activities and specifically from the blend Cytolase 104, which contains both hemicellulase and cellulase. The biopolymer levels did not change dramatically at this dosage, except again for the blend. However, in the 5X dosage study (Table I-B) a significant drop in viscosity was seen in the flask containing hemicellulase activities, and biopolymer was greatly reduced in the two flasks containing pectinase activity.

EXAMPLE II

Effect of Combining Enzymatic Activity on Substrate

Following the methods described in the Biopolymer Assay provided above and dosing at 1-2X dosage, the effect of the combination of various enzymatic activities (i.e., cellulase, pectinase and hemicellulase activity) was measured. The results are shown in Table II.

Table II

Activities	Viscosity (cp)	Biopolymer (g/l)
Control (no enzymes)	201	4.20
Sumizyme AC/Cytolase 177	150	3.34
Sumizyme AC/Sumizyme AP-2	132	1.87
Cytolase 104	98	2.48
Cytolase 123*/Sumizyme AP-2*	90	2.99

* 2X Dosage

Discussion:

By combining activities there is a slight competition or product inhibition, as seen by using the same hemicellulase with different pectinases on residual biopolymer.

EXAMPLE III

Effect of Certain Enzyme Activities on Free Galactose

The biopolymer responsible for membrane fouling is the fraction of the acidic polysaccharides or the pectins. In this experiment, hemicellulase and cellulase activities with and without pectinase added, are exposed to the soy substrate to look for galactose release. Since pectin is comprised of polymers of galacturonic acid, when hydrolysis occurs, free galactose is a by-product. Galactose release was measured following the methods described above. Data are shown in Table III below.

Table III

Activities	Time (min)	Free Galactose (g/l)*
Sumizyme AC/ Cytolase 123	30	0.028
	60	0.066
	90	0.094
Sumizyme AC/ Cytolase 123/ Sumizyme AP-2	30	3.920
	60	9.440
	90	11.000

* At time 0, free galactose = 0 mg/ml

Discussion:

As seen in Table III, the flask with pectinase added shows that free galactose release increases over time when exposed to the soy substrate.

EXAMPLE IV

Effect of Certain Enzyme Activity on Reducing Sugar Release and MW Profile

This example examines the effect of combining activities to study hydrolysis as measured by an increase in reducing equivalents and a decrease in high molecular weight polymers (>50,000MW).

Reducing sugars were measured as described in the methods above and the decrease in high molecular weight fractions and the increase in low molecular weight fractions (<50,000MW)

were based on total integration of the chromatograph peaks and normalized with respect to a control condition. 1X dosage used in this example is equivalent to the percentage of the individual enzymatic components defined for Example I and Tables I-A and I-B. Data are presented in Tables IV-A and IV-B.

Table IV-A

Cyt917 / PCL5 / Ultra-SPL Blends

Blend	Cyt917 (X)	PCL5 (X)	Ultra-SPL (X)	-HMW (%)	+R.E.^ (%)	+LMW* (X)
1	2	2	2	57	35	1.91
2	2	0	1	67	40	2.45
3	0	2	1	62	34	1.73
4	0	0	1	60	21	1.00
5	2	1	2	57	35	1.22
6	2	1	0	45	54	4.33
7	0	1	0	37	49	2.86
8	1	2	2	47	29	3.26
9	1	2	0	38	45	3.85
10	1	0	2	48	39	1.65
11	1	0	0	26	52	1.38
12	1	1	1	47	34	2.08
13	1	1	1	49	33	2.05

^ denotes increase in reducing ends as measured by the PAHBAH assay compared to a non-macerated flask.

* denotes increase in low molecular weight, defined as a multiplicity factor (X), normalized to the control (Blend 4 gives the smallest increase in low molecular weight products).

Table IV-B

Pearex / Clarex / AC/123 Blends

Blend	Pearex	Clarex	AC/123	-HMW	+R.E.^	+LMW*
1	2	2	1	32	72	5.98
2	2	1	1	34	65	10.22
3	0	2	1	30	61	6.37
4	0	0	1	21	40	1.00
5	2	1	2	36	72	7.53
6	2	1	0	35	65	6.98
7	0	1	2	29	69	8.45
8	0	1	0	30	58	7.06
9	1	2	2	35	75	6.66
10	1	2	0	34	70	5.37
11	1	0	2	32	71	12.20
12	1	0	0	27	55	11.70
13	1	1	1	35	68	7.76
14	1	1	1	36	64	7.81

^ denotes increase in reducing ends as measured by the PAHBAH assay compared to a non-macerated flask.

* denotes increase in low molecular weight, defined as a multiplicity factor (X), normalized to the control (Blend 4 gives the smallest increase in low molecular weight products).

Discussion:

In Table IV-A the effects of combining a hemicellulase/cellulase product (Cyt917) with two different pectinase products (PCL5 and Ultra-SPL) are shown. Table IV-B shows the effects of combining two different pectinases (Pearex, Clarex) with a hemicellulase/cellulase product (AC/123). The enzymes used in this treatment contain both endo- and exo-activities, i.e., ability to hydrolyze carbohydrates from within (endo-) or at the ends (exo-). The endo-activities are good for breakdown whereas exo-activity is helpful in modification of carbohydrate polymers. For example, Table IV-A shows good reductions of the high

molecular weight fractions, but not a dramatic increase in reducing equivalents, indicating that some of these combinations, are high in endo-activities, but not exo-activities. While in Table IV-B the high molecular weight fractions are not broken down dramatically, there is an increase in both reducing equivalents and low molecular weight by-products, indicating that these enzymes possess high exo-activities which hydrolyze at the ends. Such hydrolysis is limited to a point at which their activity stops. From Tables IV-A and IV-B and from knowledge of the desired biopolymer to be affected, one can select a suitable combination of enzymes that will bring about the desired result.

EXAMPLE V

Effect of Enzyme Activity on Flux Rate (Membrane Fouling)

Following the process described in the "Flux Rate" Method section above, the effect of selected enzyme activities on soy biopolymer and the subsequent flux rates through a UF membrane were measured. An objective of the present invention is to limit membrane fouling, thus maintaining a good flux rate through the ultrafiltration system. The data are shown in Table V. The results are graded as volume of permeate delivered per unit time per membrane area.

Table V

Condition (0.07% wt.)	Flux Rate (ml/min/cm^2)
Control (no treatment)	0.016
AC/123	0.024
PCL5 + AC/123	0.027
Pectinex Ultra-SPL + AC/123	0.045

Discussion:

As shown in Table V, treatment with enzymes (as compared to no treatment) increased flux rates 2.8X.

The enzyme products of microbial fermentations, particularly Bacillus protease fermentations, are routinely formulated with liquid detergent components to form a final detergent product. During the formulation step of such enzyme-containing product, gelatinous, carbohydrate-containing

precipitates are known to form. The enzyme product of soy-based fermentations, such as described herein, when formulated with liquid detergents similar to the type disclosed in US Patent 4,507,219, resulted in the formation of a gelatinous precipitate. Although the inventors do not wish the invention to be limited, it is believed that such gelatinous precipitate is due to the low available water content and highly chelating environment of these heavy-duty liquid detergent formulations (US 4,507,219), conditions which would be expected to cause the precipitation of pectic substances (R.R. Selvedran, B.J.H. Stevens and M.A. O'Neill (1985) in Biochemistry of Plant Cell Walls, Cambridge Univ. Press, New York, pp. 39-78.)

EXAMPLE VI

Effect of Macerating Enzyme Treatment on Liquid Detergent

A: Detergent Precipitate Formation:

In the present example, a precipitate was formed from a Bacillus protease (subtilisin) soy-based fermentation (the subtilisin was as described in US Patent 4,760,025 which is incorporated herein by reference) when such subtilisin was incorporated into a heavy-duty liquid detergent product. The precipitate was isolated by filtration through Whatman GFC glass fibre discs, washed with excess ethanol, dried and analyzed by 2M TFA hydrolysis, followed by GC separation of the alditol acetates (Complex Carbohydrate Corporation, Athens, GA). A representative analysis is shown below.

TABLE A

Monosaccharide	%
Rhamnose	10%
Fucose	18%
Arabinose	6%
Xylose	28%
Mannose	2%
Galactose	15%
Glucose	22%

This composition is consistent with the know pectins, pectinic acids and xyloglucans of soy (J.F. Kennedy and C.A.

White (1983) Bioactive Carbohydrates, Wiley and Sons, New York pp. 155-161).

B: Assay for the Precipitate Precursors:

The weight of this ethanol-washed precipitate was correlated to the amount of galactose released from the ethanol-precipitable carbohydrate fraction of the fermentation product (the carbohydrate fraction). Briefly, 150μℓ of sample was mixed with 150μℓ of 20% TCA (trichloroacetic acid) and centrifuged for 5 minutes in a microcentrifuge. The supernatant (150μℓ) was diluted ten fold (+1400μℓ) with ethanol, incubated at -20°C for 15 minutes and centrifuged for ten minutes as before. The precipitate was hydrolyzed with 350μℓ 10% HCl at 104°C for 75'. The products were analyzed for galactose and glucose by chromatography on a BioRad HPX87H column (0.4mL/min, 10mM H_2SO_4). As shown in Figures 3 and 4, the weight of the precipitate recovered from a representative liquid detergent correlates to the amount of galactose, but not glucose.

C: The Effect of Macerating Blend on the Amount of Precipitate Precursors:

The precipitation of this carbohydrate gel upon mixing of the fermentation product with the liquid detergent can be minimized by treatment of the initial media with macerating enzyme. The precursor analysis detailed above was performed on the products of Bacillus fermentations of the type noted in Figures 1 and 2. The variable tested was a dose of macerating enzymes (on a hemicellulase basis) (Cytolase 104) as defined in Example I. The results are indicated in Figure 5 and show clearly that a 6 fold reduction in precipitate precursors (measured as galactose) was obtained. It was surprising that although the gelatinous precipitate is characteristic of a pectinic substance, treatment with pectinase alone did not solve the gel problem. Conversely, treatment with a substantially hemicellulase-containing enzyme complex prior to fermentation resulted in marked improvement of the problem (at least 6 fold reduction).

WHAT IS CLAIMED IS:

1. A process for improving carbohydrate-rich fermentation of a desired product and for improving the recovery of the desired product, the process comprising:

a) contacting for a sufficient time a carbohydrate-rich fermentation medium with an effective amount of a macerating enzyme complex comprising hemicellulase activity, cellulase activity, and/or pectinase activity, wherein the enzymatic activities are present in an amount sufficient to improve fermentation handling and to improve downstream recovery of the desired product as compared to fermentation without the macerating enzyme complex;

b) inoculating the fermentation medium of step a);

c) fermenting the medium of step b) under suitable conditions; and

d) recovering the desired product.

2. A process of claim 1 wherein the fermentation medium is soymeal.

3. A process of claim 1 wherein the desired product is a protease.

4. A process of claim 1 wherein the fermentation is run at a temperature of between about 30°C to about 40°C and a pH of between about 6.5 to about 8.

5. A process of claim 1 wherein the recovery of the desired product comprises centrifugation and ultrafiltration.

6. A process of claim 1 wherein the enzyme complex comprises cellulase and hemicellulase activity, and wherein the enzyme complex reduces the viscosity of the carbohydrate-rich fermentation and/or increases the overall mass transfer (KLa) in the carbohydrate-rich fermentation.

7. A process of claim 1 wherein the enzyme complex comprises substantially only pectinase activity, wherein said complex increases the flux rate during ultrafiltration downstream of a carbohydrate-rich fermentation.

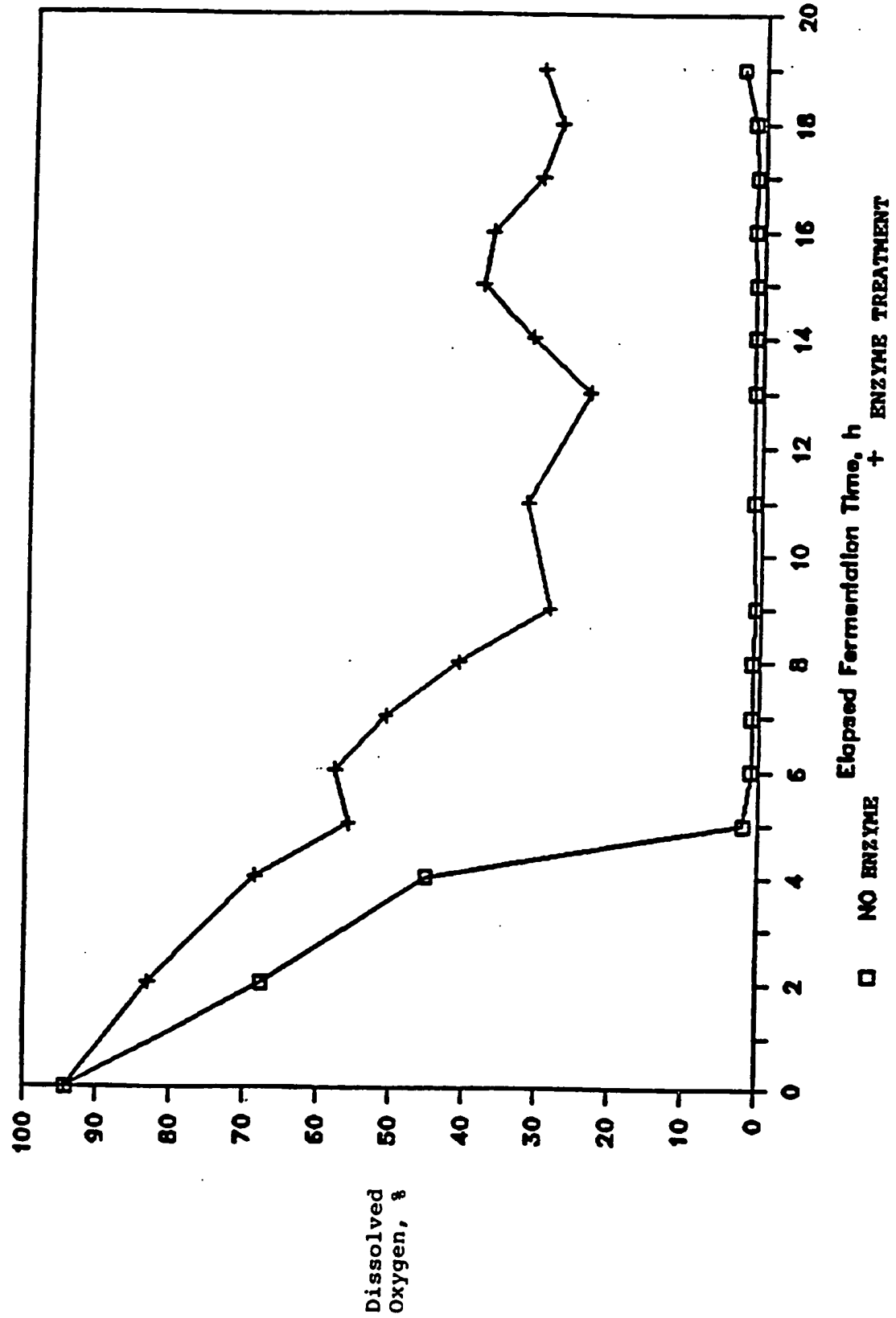
Dissolved Oxygen, %
100
90
80
70
60
50
40
30
20
10
0
0
2
4
6
8
10
12
14
16
18
20
Elapsed Fermentation Time, h
□ NO ENZYME
+ ENZYME TREATMENT

FIGURE 1

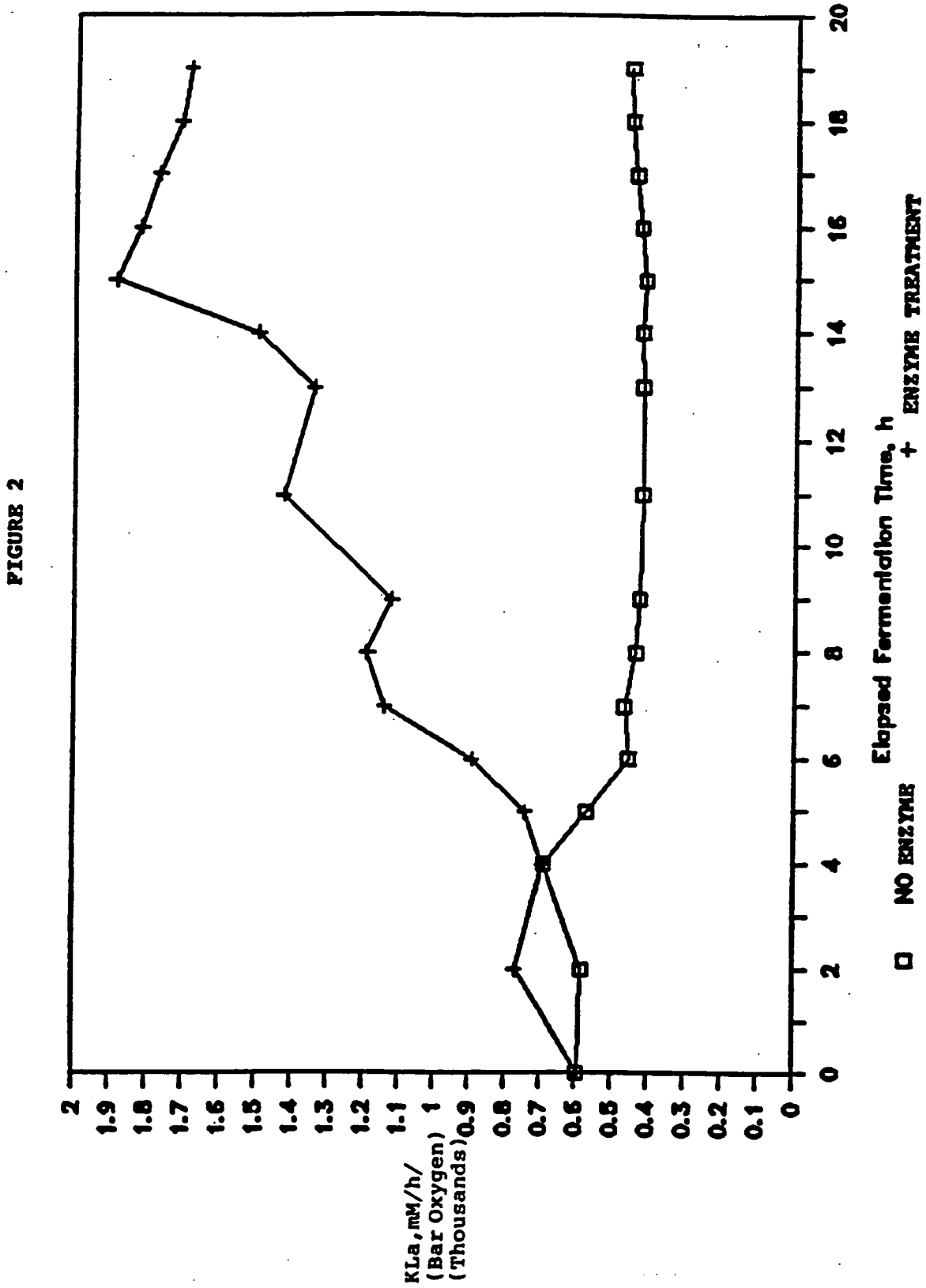
KLa, mM/h/
(Bar Oxygen)
(Thousands)
2
1.9
1.8
1.7
1.6
1.5
1.4
1.3
1.2
1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
2
4
6
8
10
12
14
16
18
20
Elapsed Fermentation Time, h
□ NO ENZYME
+ ENZYME TREATMENT

FIGURE 2

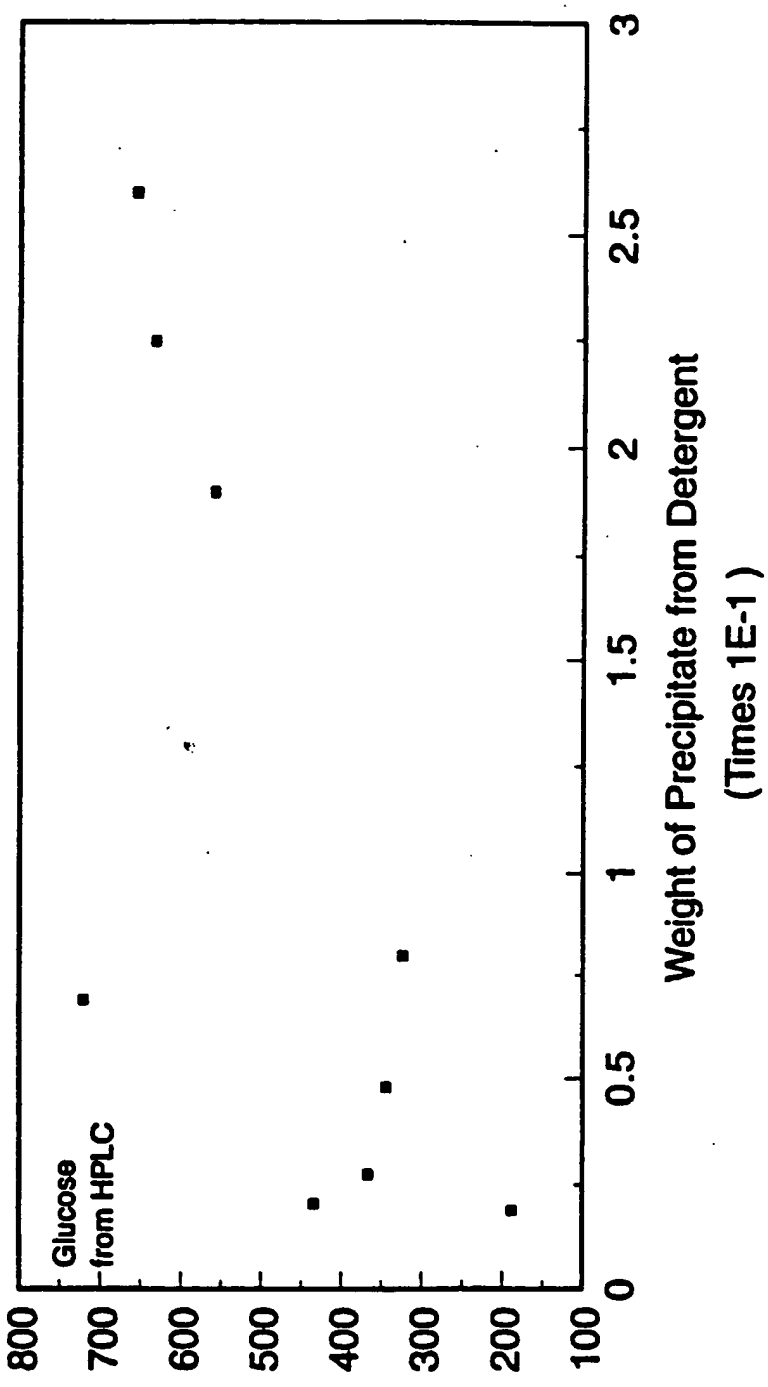
Glucose from HPLC
800
700
600
500
400
300
200
100
0
0.5
1
1.5
2
2.5
3
Weight of Precipitate from Detergent
(Times 1E-1)

figure 3

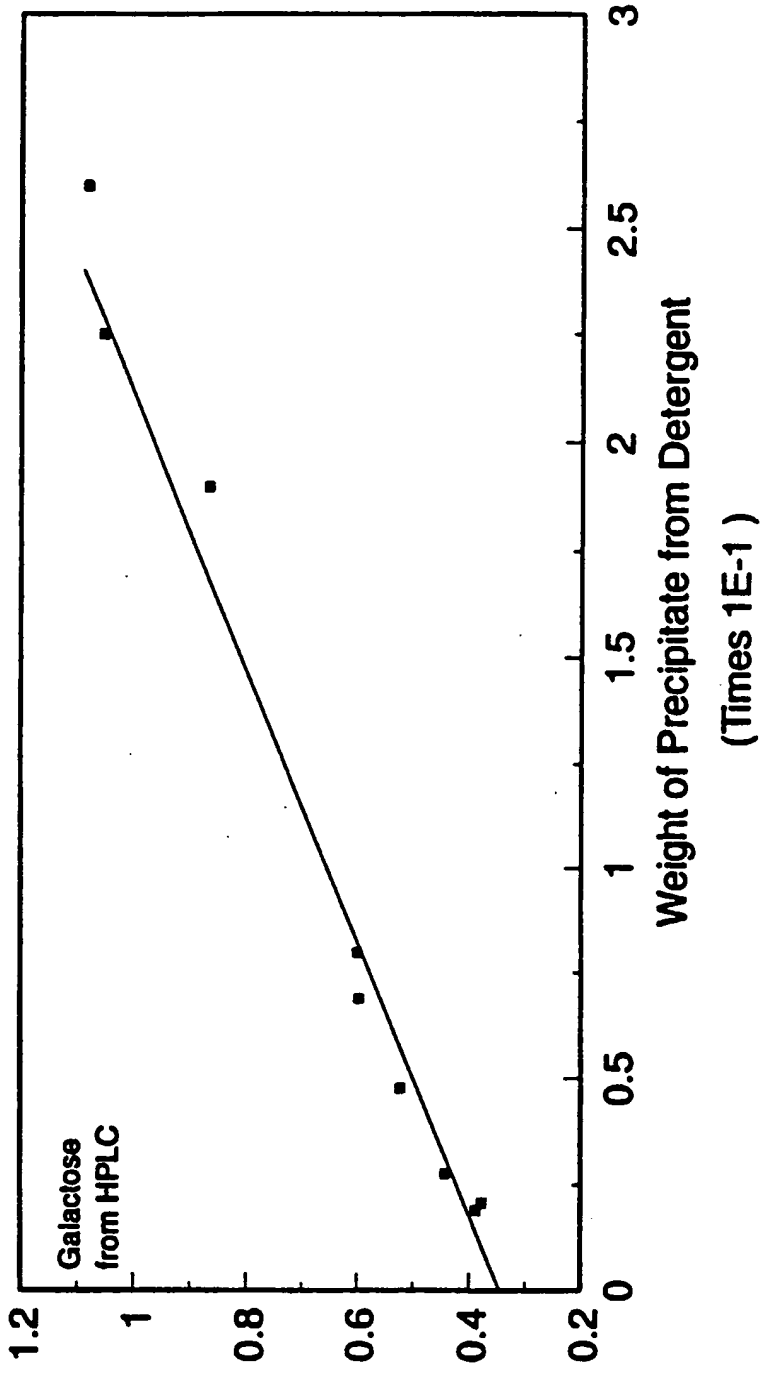
Galactose
from HPLC
1.2
1
0.8
0.6
0.4
0.2
0
0.5
1
1.5
2
2.5
3
Weight of Precipitate from Detergent
(Times 1E-1)

figure 4

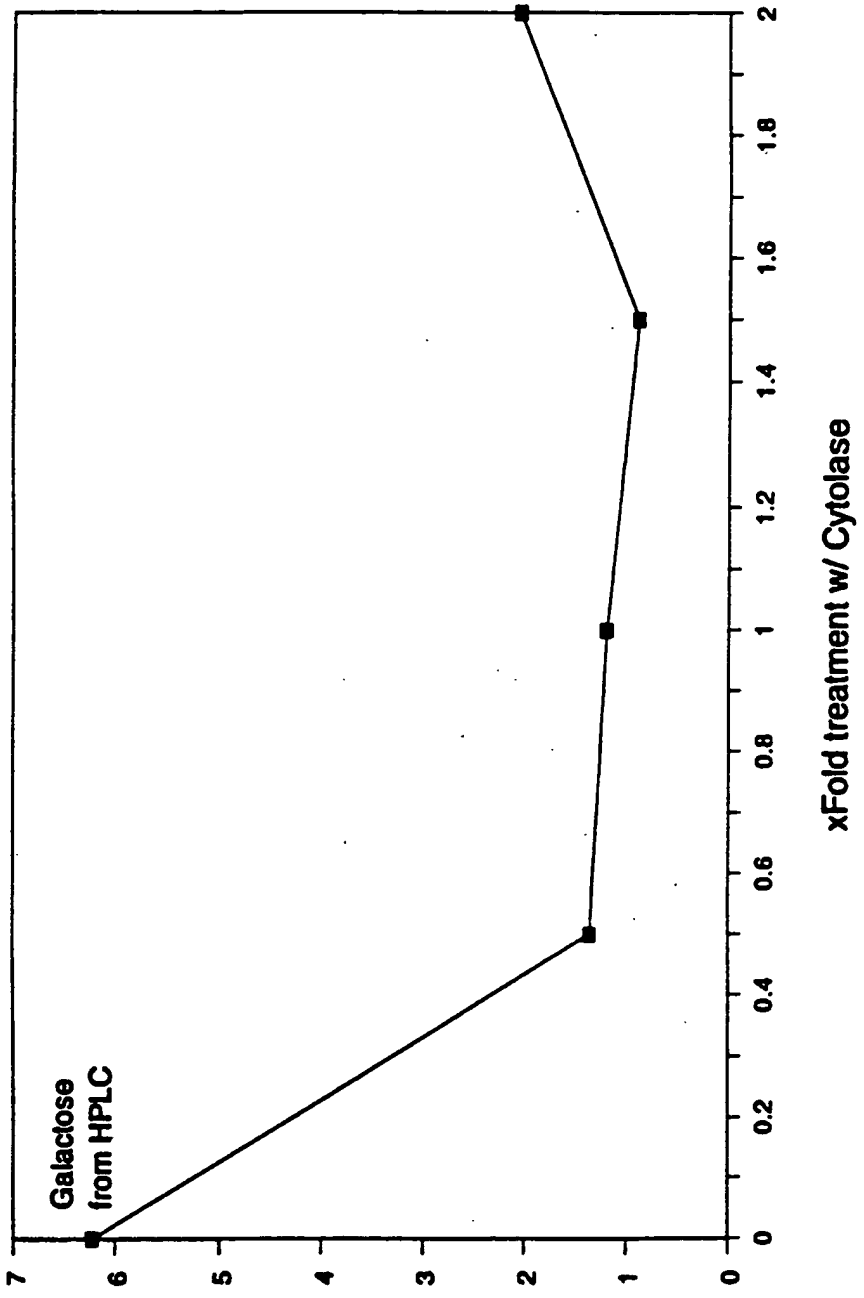
Galactose from HPLC
xFold treatment w/ Cytolase
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
1.8
2
7
6
5
4
3
2
1
0

figure 5

INTERNATIONAL SEARCH REPORT

Interna ul Application No
PCT/US 94/08534

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N1/22 C12N1/38 C12N1/20 C12N9/56 //C12N9/42, C12N9/24

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	EP,A,0 329 559 (SANOFI S.A.) 23 August 1989 see the whole document ---	1-7
Y	US,A,4 056 636 (MUELLER H.) 1 November 1977 see column 3 lines 28,58 see claim 1 ---	1-7
Y	FR,A,2 400 062 (SORG E.) 9 March 1979 see the whole document ---	1
Y	GB,A,2 139 471 (NOVO INDUSTRI A/S) 14 November 1984 see the whole document ---	1
A	-/--	6

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
15 November 1994	01. 12. 94

Name and mailing address of the ISA	Authorized officer
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INTERNATIONAL SEARCH REPORT

Intern: al Application No
PCT/US 94/08534

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	CHEMICAL ABSTRACTS, vol. 91, no. 17, 22 October 1979, Columbus, Ohio, US; abstract no. 139143w, SIVERS ET AL. 'Enzymic lysis of acid hydrolysates of plant residues' page 519 ;column 139153 ; see abstract & MIKROBIOL. ZH., vol.41, no.4, 1979, KIEV pages 363 - 366 SIVERS ET AL.	1
A	BE,A,675 878 (CESKOSLOVENSKA AKADEMIE VED) 16 June 1966 see page 2 -last paragraph see page 5, paragraph 3	1
A	EP,A,0 388 115 (NALCO CHEMICAL COMPANY) 19 September 1990 see the whole document	1
A	CHEM. ENG. J., vol.44, no.3, 1990 pages B51 - B55 VLAEV ET AL. 'Rheology and oxygen transfer in starch suspensions during high temperature (35 Deg) alpha-amylase fermentation-KLa determination in pseudoplastic starch suspension during Bacillus licheniformis fermentation' see the whole document	6

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

Interna ·l Application No
PCT/US 94/08534

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		DE-D-	68915568	07-07-94
		JP-A-	1247086	02-10-89
		US-A-	5187081	16-02-93
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		AU-B-	567928	10-12-87
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		CA-A-	1239829	02-08-88
		CH-A-	664874	15-04-88
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		FR-A-	2544178	19-10-84
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		JP-A-	3000193	07-01-91